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Ubiquitin-Associated domain of Mex67 synchronizes recruitment of the mRNA export machinery with transcription

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Abstract

The mRNA nuclear export receptor Mex67/Mtr2 is recruited to mRNAs through RNA-binding adaptors including components of the THO/TREX complex that couple transcription to mRNA export. Here we show that the ubiquitin-associated (UBA) domain of Mex67 is not only required for proper nuclear export of mRNA but also contributes to recruitment of Mex67 to transcribing genes. Our results reveal that the UBA domain of Mex67 directly interacts with polyubiquitin chains and with Hpr1, a component of the THO/TREX complex, which is regulated by ubiquitylation in a transcription-dependent manner. This interaction transiently protects Hpr1 from ubiquitin/proteasome-mediated degradation and thereby coordinates recruitment of the mRNA export machinery with transcription and early mRNP assembly.

Introduction

Concomitantly to their transcription, nascent transcripts are loaded with mRNA binding proteins implicated in the processing and packaging of mRNA into stable and export competent mRNPs. The production of mature mRNPs involves 5' capping, splicing, 3'end cleavage/polyadenylation. All these co-transcriptional but biochemically distinct reactions are tightly coupled and coordinated by the RNA polymerase II C-terminal domain (CTD), which acts as a recruitment platform for the different processing machineries (1-5).

Fully mature and correctly packaged yeast mRNPs are released from the transcription site and transported into the cytoplasm by the heterodimeric export receptor Mex67/Mtr2 (or TAP/p15 in metazoan), which promotes their translocation through the nuclear pore complexes (NPC) via direct interactions with FG-nucleoporins (6). Mtr2 promotes the interaction of Mex67 with the NPC (7). The adaptor protein Yra1/REF contributes to mRNA export by facilitating the binding of Mex67 to the mRNP (8, 9). Yra1/REF and its partner Sub2 (UAP56 in metazoan) become associated with nascent transcripts during transcription elongation (10, 11). Yra1 and Sub2 co-purify with THO, a tetrameric complex associated with the transcription machinery, to form the TREX complex proposed to link mRNA transcription and export (12). Constituants of THO include Hpr1, Tho2, Mft1 and Thp2 (12, 13), and evidence supports the notion that early recruitment of Sub2 is promoted through its direct interaction with Hpr1. Sub2 may then in turn facilitate the loading of Yra1 to mRNA (11, 12, 14, 15).

Loss of any of the four THO components impairs transcription elongation, genome stability and mRNA export (12, 13). The current view is that THO primarily contributes to efficient mRNP assembly by promoting correct loading of mRNA binding proteins. In THO mutants, the production of improperly packaged mRNP complexes results in the formation of DNA-RNA hybrids (R-loops), which interfere with RNA polymerase II processivity and

transcription elongation and consequently increase transcription-dependent recombination events (16, 17). Furthermore, the absence of THO components induces retention and eventually degradation of malformed transcripts, i.e. heat-shock *HSP104* mRNAs, at or close to the transcription site by nuclear surveillance mechanisms (18).

Previous studies have indicated that the ubiquitin pathway is involved in regulation of nuclear transport of both poly(A)$^+$RNA and proteins (19). In particular, Tom1 and Rsp5, two ubiquitin ligases from the HECT family have been shown to play a role in nuclear export of poly(A)$^+$RNA in *S. cerevisiae* (20-23). We recently reported that Hpr1, a component of the THO complex is poly-ubiquitylated both *in vitro* and *in vivo* by Rsp5 prior to its degradation by the 26S proteasome. Hpr1 turnover, which is more active at 37°C, appeared linked to on-going RNA-polymerase II–dependent transcription whereas the other members of the THO complex such as Mft1 or Thp2 were not affected in similar conditions (24). Hpr1 thus represents a key factor whose stability controls the integrity and activity of the whole THO complex. Paradoxically, *HPR1* deletion, but also Hpr1 stabilization by inactivation of Rsp5 correlated with a poly(A)+ RNA nuclear export defect, suggesting that tight control of both expression and active ubiquitin-dependent turnover of Hpr1 is required for proper transport function.

The large array of cellular processes involving ubiquitin modification is likely mediated through recognition of ubiquitin moieties by effectors containing ubiquitin-binding domains. Several families of ubiquitin-interacting motifs have been recently identified including UBA (Ubiquitin Associated), the most frequent ubiquitin-binding motif, UIM (Ubiquitin interacting motif), CUE (coupling of ubiquitin conjugation to endoplasmic reticulum), NZF (Npl4 Zn finger), UEV (ubiquitin E2 enzyme variant) (reviewed in (25). Structural and molecular features of UBA-ubiquitin interactions and ubiquitin linkage selectivity have been analyzed in a restricted number of UBA-containing proteins,

exemplified by yeast Rad23 or its human ortholog hHR23A, leading to the general assumption that UBA domains preferentially interact with Lys48-linked poly-ubiquitylated proteins (26-32). In contrast to this current view, a recent study on 30 distinct UBA motifs revealed that, *in vitro*, a UBA motif may or not be selective for the ubiquitin linkage but may also display no measurable affinity for ubiquitin (33). In addition, the idea emerges that the intra-molecular environment of a UBA domain may influence the specificity of the UBA domain (33), but ubiquitin-binding domains described to date have not been reported to display any particular specificity for a ubiquitylated protein. Interestingly the yeast mRNA export receptor Mex67 as well as its metazoan counterpart TAP harbor a UBA domain in their C-terminus that participates in the interaction with FG-nucleoporins at nuclear pores while their N-terminal domain binds mRNP via RNA-binding adaptors (8, 9, 34-37). The UBA domain of Mex67 consists of the characteristic triple helix bundle plus an additional fourth helix. Mex67 is so far the only nuclear transport factor containing an ubiquitin-associating motif but its role in connecting mRNA nuclear export to regulation by ubiquitin modification remains to be determined.

Here we show that Mex67 is recruited to transcribing genes and that its UBA domain contributes to its recruitment. Lack of recruitment correlates with a defect in mRNA export. Our data show that the UBA domain of Mex67 not only recognizes ubiquitin and polyubiquitylated proteins but also physically interacts with Hpr1. In addition, an excess of UBA-Mex67 or the absence of UBA (Mex67-ΔUBA), respectively, induces a decrease or an increase in the rate of degradation of Hpr1, consistent with a role for the UBA-Mex67 domain in transient protection of ubiquitylated Hpr1 from degradation by the 26S proteasome. The Mex67-Hpr1 interaction may contribute to the appropriate coordination of the different steps of mRNP biogenesis

Results and Discussion

To precisely characterize the function of the UBA domain of Mex67 (UBA-Mex67) in mRNA nuclear export, the subcellular distribution of mRNA was analyzed upon deletion or overexpression of the UBA-Mex67 domain. Strains were constructed that lacked the chromosomal *MEX67* gene and expressed HA-tagged wild-type Mex67 (Mex67-3HA) or Mex67ΔUBA (*mex67ΔUBA-3HA*) from a plasmid. Western blotting with anti-HA antibodies confirmed that the wild-type protein (encoding amino acids 1-599) and the mutant protein (encoding amino acids 1-542) were expressed to comparable levels (not shown). In agreement with previous results reported by (7), deletion of the UBA-Mex67 domain rendered cells thermosensitive for growth at 37°C (not shown). It should be noted that the Mex67ΔUBA protein used in this former study lacked the C-terminal 75 amino acids whereas Mex67ΔUBA-3HA only lacks the last 57 amino acids strictly corresponding to the UBA domain. Consistent with its growth phenotype, the *mex67ΔUBA*-3HA strain displayed a weak mRNA export defect at 23°C (8% of the cells with nuclear accumulation of poly(A)+RNA) whereas poly(A)+RNA accumulated in the nucleus of 74% of the cells after a 1h shift to the restrictive temperature. In contrast, only 1% of the Mex67-3HA cells presented nuclear accumulation of poly(A)+RNA under the same experimental conditions (Figure 1A). Similarly, yeast cells over-expressing the Lex-UBA chimeric protein clearly accumulated poly(A)+RNA in their nucleus after a 2h-shift to 37°C compared to 23°C or to control cells (Figure 1C). *GAL1* mRNA, whose transcription was induced for 90 min in galactose at 23°C mainly accumulated within a nuclear dot at or close to the site of transcription and was poorly detected in the cytoplasm of *mex67ΔUBA*-3HA cells whereas this mRNA was distributed throughout Mex67-3HA cells with a detectable but much weaker dot staining, indicating a role for the UBA-Mex67 domain in the release and export of this specific transcript (Figure 1B). These results indicate that the UBA domain is required for Mex67 to ensure proper

nuclear export function. The more pronounced effect of UBA deletion both on cell growth and on nuclear export at 37°C and the capacity of Mex67ΔUBA to bind nucleoporins *in vitro* (7) suggest that the UBA domain may play a role distinct from its nuclear pore complex targeting function.

Mex67/Mtr2 is recruited to mature mRNPs through RNA-binding adaptors that interact with its N-terminal domain. To determine whether the UBA-Mex67 domain may function in an earlier more upstream event of mRNA biogenesis, the ability of Mex67 to be recruited to actively transcribed genes was analyzed by chromatin immunoprecipitation (ChIP) of the galactose inducible *GAL10* gene (Figure 2A) and the constitutively expressed *PMA1* gene in cells grown respectively in galactose and glucose (Figure 2B). Our results show that Mex67 becomes associated with the *PMA1* gene and with *GAL10* when cells are grown in galactose indicating that recruitment of Mex67 is transcription-dependent (Figure 2). Mex67 was enriched in the middle of both *PMA1* and *GAL10* genes showing an association profile similar to that observed earlier for the THO complex component Hpr1 and the mRNA adaptor Yra1 (Figure 2A and B; (11)). Interestingly, absence of the UBA domain resulted in the clear decrease of co-transcriptional recruitment of Mex67 all along *GAL10* and *PMA1* genes (Figure 2 A and B). However neither CTD nor Yra1 recruitment was significantly affected in the mutant, indicating that the reduced recruitment of Mex67-ΔUBA was not a consequence of a transcriptional defect and that the co-transcriptional recruitment of Mex67-3HA was mainly due to its UBA domain (Figure 2A and B). Despite a normal recruitment of both CTD and Yra1 in *mex67ΔUBA*-3HA cells, we observed a decrease of Hpr1 on *GAL10* gene in these cells, again suggesting that UBA-Mex67 could interfere with the function of some factors involved in the coupling between transcription and mRNA export (Figure 2B and see below).

To dissect the mechanisms responsible for the roles of UBA-Mex67, partners of this domain were searched using a two-hybrid screen. This strategy allowed the identification of a 203 amino acid C-terminal fragment of Hpr1 (aa 548-752) that interacted with UBA-Mex67, as well as with full-length Mex67. No interaction was observed with Mex67 lacking its UBA domain (Figure 3A). The specificity of the interaction between Hpr1 and Mex67 was confirmed using UBA-Rad23 as well as an Hpr1 molecule deleted of its C-terminal fragment (Hpr1(1–547)). Interestingly, no significant interaction was measured between UBA-Mex67 and Mft1 or Thp2, suggesting that the interaction between Mex67 and Hpr1 does not involve the other members of the THO complex (Figure 3A). In agreement with the 2-hybrid results, recombinant purified His-tagged UBA-Mex67 could interact *in vitro* with GST-Hpr1. This interaction occurred through the C-terminal fragment of Hpr1 (GST-Hpr1 (548-752)) corresponding to the 2-hybrid fragment, whereas a smaller C-terminal fragment (Hpr1 (652-752)) was not sufficient to significantly bind UBA-Mex67 in vitro (Figure 3B). Finally, co-immunoprecipitation was equally observed between Hpr1-HA and Mex67, and Thp2-HA and Mex67, indicating that Mex67 can interact with Hpr1 in the context of the THO complex in intact cells when expressed at physiological levels (Figure 3C). However, no co-immunoprecipitation between Mex67 and Thp2-HA could be observed in absence of Hpr1 (Figure 3C) thus demonstrating that this interaction is also mediated by Hpr1 *in vivo*. Together, these results clearly show that UBA-Mex67 promotes binding of Mex67 to Hpr1 whereas an unrelated UBA domain cannot provide this function.

Based on a few well-studied examples such as UBA-Rad23, UBA folds are believed to interact with and either expose or protect poly-ubiquitylated substrates to/from the 26S proteasome (38-40). However, a recent study on 30 distinct UBA domains revealed that about 30% of UBA domains do not bind mono- or polyubiquitin chains (33). The ability of UBA-Mex67 to interact with ubiquitin moieties was therefore carefully analyzed both *in vivo* and *in*

vitro. GST pull-down experiments using the GST-Mex67/Mtr2 dimer revealed that the mRNA export receptor was able to bind poly-ubiquitylated cellular proteins accumulated upon treatment with the proteasome inhibitor MG132 (Figure 4A). *In vitro* titration experiments indicate that GST-Mex67/Mtr2 clearly interacts with ubiquitin with a higher affinity for tetra-ubiquitin (Kd=5.9+/-0.7 μM) than for monoubiquitin (Kd=22.7+/-4.2 μM), values comparable with those reported for other proteins harboring a UBA domain (33, 39). Importantly, deletion of the UBA domain abolished the ability of Mex67 to bind ubiquitin both *in vivo* and *in vitro* (Kd>400 μM; Figure 4A and 4B). These results therefore demonstrate that Mex67 binds poly-ubiquitylated cellular proteins through its UBA domain.

We recently reported that Rsp5, a WW domain-containing ubiquitin ligase involved in the control of mRNA export (22, 24) poly-ubiquitylates Hpr1 prior to its degradation by the proteasome in a temperature and transcription-dependent fashion. The lysine-rich C-terminal domain of Hpr1 is not only required for proper ubiquitylation and degradation of Hpr1 (24) but also for interaction with UBA-Mex67 (Figure 3A and B), suggesting that ubiquitylation of Hpr1 may influence its binding to UBA-Mex67, which might in turn affect Hpr1 degradation. To examine the potential effect of the UBA-Mex67 domain on Hpr1 turnover, Hpr1 levels were analyzed when the UBA domain was either absent or present in excess. The data show that loss of UBA-Mex67 resulted in faster degradation of Hpr1 after a shift to 37°C, whereas overexpression of Lex-UBA-Mex67 was able to protect Hpr1 from degradation at 37°C when compared to overexpression of the Lex-UBA-Rad23 fusion protein (Figure 4C). Interfering with the interaction between Hpr1 and Mex67 thus clearly affects the degradation of Hpr1. To distinguish whether the interaction of Hpr1 with UBA-Mex67 prevents Hpr1 degradation by inhibiting Hpr1 ubiquitylation or rather by protecting the polyubiquitin chain from the proteasome, polyubiquitin-conjugated species of Hpr1 were analyzed upon overexpression of UBA-Mex67 or UBA-Rad23. For this purpose, His6-tagged ubiquitin and UBA domains

were overexpressed in *cim3.1* temperature-sensitive mutants, impaired in proteasomal activity and grown at the restrictive temperature (24). Affinity purification followed by Western blot analysis showed that polyubiquitylated species of Hpr1 accumulated upon over-expression of UBA-Mex67 compared to UBA-Rad23 (Figure 4D). In agreement with these results, we found that, *in vitro*, the affinity of UBA-Mex67 for ubiquitin was strongly increased upon binding to Hpr1 (Hobeika et al., unpublished observations). The specificity of the interaction between UBA-Mex67 and Hpr1 thus allows UBA-Mex67 to interfere with the ubiquitin/proteasome-mediated degradation of Hpr1 by transiently protecting ubiquitylated Hpr1 from the 26S proteasome.

To determine whether the interaction between UBA-Mex67 and ubiquitylated Hpr1 not only affects Hpr1 turnover but also contributes to the co-transcriptional recruitment of Mex67 and mRNA export, these functions were analyzed in a mutant affected in Hpr1 ubiquitylation. The interaction between Rsp5 E3 ligase and Hpr1 most likely involves the recognition of the LPxY motif of Hpr1 (amino-acids 335-338, Figure 5A) by the second and third WW repeats of Rsp5 (22, 24). Indeed mutation of tyrosine 338 to alanine (*hpr1-Y338A*) slowed down Hpr1 turnover at 37°C confirming that ubiquitin-dependent degradation of Hpr1 is affected by a mutation in the Rsp5-binding motif LPxY (Figure 5A). This partial block in Hpr1 ubiquitylation also resulted in a defect of *GAL1* mRNA nuclear export illustrated by a clear and reproducible accumulation of *GAL1* mRNA within a marked nuclear dot and a lack of cytoplasmic staining after a 30 minute shift to galactose at 30°C in a majority of *hpr1-Y338A* cells compared to the wt cells (Figure 5B). However, *GAL1* transcripts were detected in the cytoplasm after longer time points indicating that this partial defect most likely results from a delayed release from the transcription site rather than an export defect *per se*. Accordingly, this ubiquitylation defect led to a 40% decrease of the co-transcriptional recruitment of Mex67 to the *GAL10* gene at 25°C. This effect did not result from a decreased

transcription of the gene nor a defect in *hpr1-Y338A* recruitment or THO complex formation and recruitment, as measured by the association of CTD, Hpr1 and Thp2 respectively (Figure 5C). Notably, the *hpr1-Y338A* mutation resulted in a slight increase of CTD and Hpr1 recruitment on *GAL10* whereas recruitment of Thp2 remained unchanged suggesting that Hpr1 ubiquitylation could influence the THO complex integrity to some extent. Together these data are consistent with the RNA-independent early binding of Mex67 (41) and further support the view that ubiquitylation of Hpr1 facilitates co-transcriptional recruitment of Mex67 and mRNA export by mediating an interaction with the UBA domain of Mex67.

Hpr1 is a key component of the THO complex whose stability is likely to control the integrity and activity of the whole THO complex (12, 24). Because ubiquitylation of Hpr1 depends on active transcription, the UBA-Mex67-mediated recruitment of Mex67 and protection of Hpr1 are likely to occur during transcription itself and may contribute to increasing the local concentration of Mex67 in the vicinity of transcribed genes, and to coordinating THO complex metabolism and mRNA export. In other words, ubiquitin-mediated degradation does not appear to be the "raison d'être" of this modification but a consequence of the accomplished assembly/recruitment function. The mechanisms described here represent the first example of the role of poly-ubiquitylation in the coordination between transcription and nuclear export through the tight control of the transport machinery assembly, disassembly and degradation. The involvement of at least another ubiquitin ligase Tom1 (20, 21), in the regulation of mRNA export, leads us to suspect that such a mechanism is not likely to be unique and could be used by eukaryotic cells to organize and control the chronology of concerted molecular events.

Experimental Procedures

Plasmids and Cloning. See Supplementary informations

FISH experiments. DF5 strain transformed with p426GAL1 plasmid encoding the fusion protein Lex-UBA-Mex67 or the empty vector were grown in YEP / 2% raffinose / 0.02% glucose medium at 23°C. The expression of Lex-UBA protein was induced by addition of 2% galactose over-night at 23°C. At OD_{600} = 0.8 cells were shifted to 37°C for 2 h. These cells were then analyzed by *in situ* hybridization (FISH) using Cy3 labeled oligo dT(50) performed as previously described (22, 43). Poly(A)$^+$ mRNA *in situ* hybridization in Mex67-3HA and *mex67-ΔUBA*-3HA strains was performed using Cy3 labeled oligo dT(50) on cells grown at 23°C or heated for 1 hour at 37°C. Results were quantified by counting, in a blind experiment, the number of cells accumulating poly(A)$^+$ mRNA in the nucleus within a total of 100 cells per each condition. *GAL1* mRNA *in situ* hybridization in Mex67-3HA and *mex67-ΔUBA*-3HA or in Hpr1 wt and *hpr1Y338A* strains was performed on cells treated for 90 min with galactose at 25°C or 30 min with galactose at 30°C, respectively, using six Cy3-internally-labelled 50mer oligonucleotide probes.

Chromatin immunoprecipitation (ChIP) analysis. See Supplementary information.

Purification of 6His-tagged Ubiquitin-Hpr1 conjugates. *cim3.1* ts cells expressing Hpr1-HA were transformed with a plasmid encoding 6His-ubiquitin under the *CUP1* promoter (46) and a plasmid encoding Lex-UBA-Mex67 or Lex-UBA-Rad23 from the ADH promoter. Cells were grown on selective media supplemented with 0.1mM of CuSO4 and shifted to 37°C for 4 h. Purification was performed essentially as described previously (24) from 50

OD_{600} of cells (2.3 mg of total proteins) and modified Hpr1 was detected using anti-HA antibody (Babco).

Co-Immunoprecipitation experiments. Yeast cells expressing Hpr1-HA or Thp2-HA were grown up to an OD_{600}= 1.2. Cells were harvested and lysed at 4°C with glass beads in ice-cold IP buffer. The lysate was centrifuged for 30 min at 13000g. The supernatant was incubated with protein G–Sepharose beads (Amersham) and anti-Mex67 or preimmune antibodies for 2h at 4 °C. Beads were then washed with IP buffer and bound proteins were eluted by heating samples at 95°C for 5 min in Laemmli sample buffer before Western-blot analysis using anti-Mex67 (24) or anti-HA antibodies (Babco).

Fluorescence titration experiments: Binding of Mex67, Mex67ΔUBA and Rad23 to ubiquitin and tetra ubiquitin was monitored by steady-state fluorescence spectroscopy as previously described (27). Fluorescence titrations were performed in 20 mM Tris-HCl (pH: 7.2), 150 mM NaCl, 2 mM EDTA, 1% glycerol, at 25 °C. Fluorescently labelled ubiquitin and tetra-ubiquitin were prepared and purified as described (27). A fixed concentration of fluorescently labelled ubiquitin or tetra-ubiquitin (1 μM) was titrated with increasing concentrations of Mex67, Mex67ΔUBA, Rad23 and Mtr2 proteins. Fluorescence was measured at 460 nm upon excitation at 340 nm. Upon binding to either Rad23 or Mex67, the fluorescence of mono or tetra-ubiquitin increased by a factor of 2 -3. The titration curves were fitted according to a quadratic equation using Grafit Software (Erithacus Software Ltd) (47).

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Figure legends

Figure 1. The UBA domain of Mex67 contributes to its mRNA export activity (A) Subcellular localization of poly(A)+ RNA was analyzed by FISH using oligodT Cy3 as probe in Mex67-3HA or in *mex67ΔUBA*-3HA shuffle strains grown over-night at 23°C and then shifted to 37°C for 1h. (B) *GAL1* mRNA was analyzed by FISH using a specific probe in Mex67-3HA or *mex67ΔUBA*-3HA shuffle strains after a 30 min induction in galactose at 23°C. (C) Cells expressing Lex or Lex-UBA Mex67 under the control of a galactose inducible promoter were grown over-night in galactose containing medium at 23°C and then shifted to 37°C for 2h prior to FISH analysis using oligodT Cy3 as probe.

Figure 2. The UBA domain of Mex67 contributes to its co-transcriptional recruitment. (A) ChIP experiments on *GAL10* gene were performed with extracts prepared from Mex67-3HA or *mex67ΔUBA*-3HA strains shifted to galactose for 2h using indicated antibodies. (B) ChIP experiments on *PMA1* gene were performed with extracts prepared from Mex67-3HA or *mex67ΔUBA*-3HA strains using antibodies against HA or RNA polymerase II C-terminal domain (CTD). Each immunoprecipitation was repeated at least from three different extracts. Error bars correspond to standard deviations.

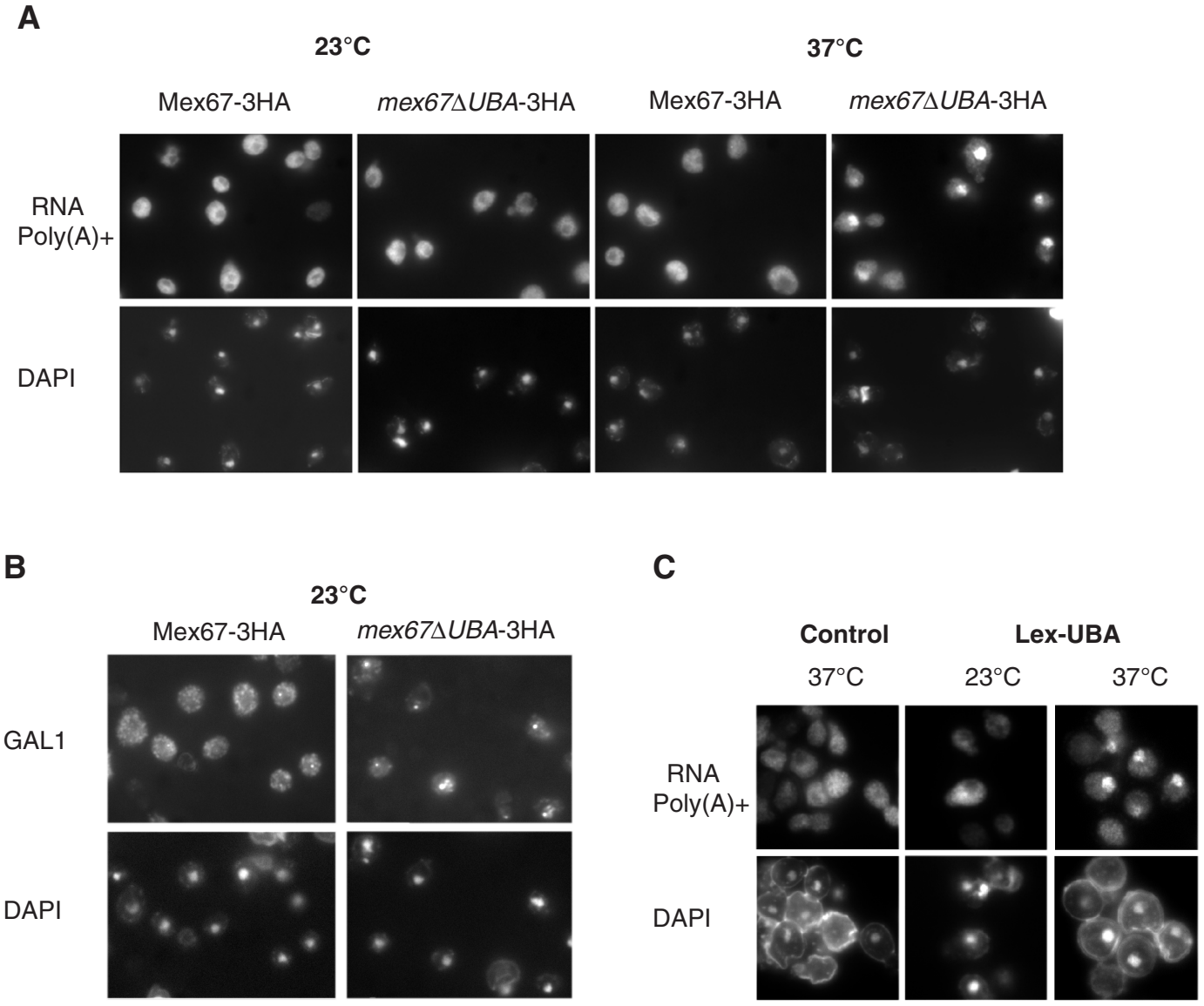
Figure 3. The UBA domain of Mex67 interacts with Hpr1. (A) Two-hybrid analysis of the Lex-Mex67 baits versus Gal4-Hpr1 derived preys. (B) Purified recombinant UBA-Mex67 and GST-Hpr1 fusion proteins were mixed (input) and complexes were analyzed after purification on glutathione-sepharose beads (bound). (C) *In vivo* Mex67/THO complex interaction was analyzed by co-immunoprecipitating HA-tagged Hpr1 with anti-Mex67 antibodies or

alternatively HA-tagged Thp2 with anti-Mex67 antibodies in Thp2-HA (*HPR1*) or *Δhpr1*/Thp2-HA *(Δhpr1)* strains. Pre-immune serum (IP mock) was used as negative control.

Figure 4. The UBA domain of Mex67 interacts with polyubiquitin chains and prevents proteasome-mediated degradation of Hpr1. (A) Pull-down assays using the indicated GST fusion recombinant proteins and extracts from *Δerg6* cells +/- MG132. (B) Dissociation constants of Mex67-derived GST fusion proteins and Rad23 for mono- and tetra-ubiquitin. The association with ubiquitin was measured *in vitro* by monitoring the fluorescence enhancement of fluorescently-labeled ubiquitin. (C) The stability of Hpr1-HA was analyzed in cells (YFS 1748) transformed with a pLex10-UBA-Mex67 or a pLex10-UBA-Rad23 plasmid and collected before (0) or after a shift at 37°C for 15 min in the absence (-CX) or the presence (+CX) of cycloheximide (left panel) or alternatively in Mex67-3HA or in *mex67ΔUBA*-3HA shuffle strains shifted to 37°C in the presence of cycloheximide for different incubation times (right panel). (D) Ni-Purified 6His-ubiquitin conjugated forms of Hpr1-HA from *cim3.1* cells transformed with pYEp96-6His-Ub and a p426ADH-Lex-UBA-Mex67 or a p426ADH-Lex-UBA-Rad23 plasmid and shifted to 37°C during 4 h. The purified material was examined by Western blotting with an anti-HA antibody.

Figure 5. Ubiquitylation of Hpr1 is required for proper mRNA export and co-transcriptional recruitment of Mex67. (A) Mutation Y338A within the Rsp5-binding site of Hpr1 slowed-down the turnover of Hpr1 (B) *GAL1* mRNA localization was analyzed by FISH using a specific probe in Hpr1wt or *hpr1Y338A* mutant strains after a 30 min induction in 2% galactose at 30°C. (C) The association of Mex67 with the 5', middle, 3' and 3'UTR regions of the *GAL10* gene was analyzed by ChIP using anti-Mex67, anti-CTD, anti-Hpr1 or anti-HA antibodies and extracts from Hpr1wt/Thp2-HA or *hpr1Y338A*/Thp2-HA cells induced with

2% galactose for 1h at 25°C. Values correspond to the mean of three independent experiments.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23°C
37°C
Mex67-3HA
mex67ΔUBA-3HA
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mex67ΔUBA-3HA
RNA Poly(A)+
DAPI
B
23°C
Mex67-3HA
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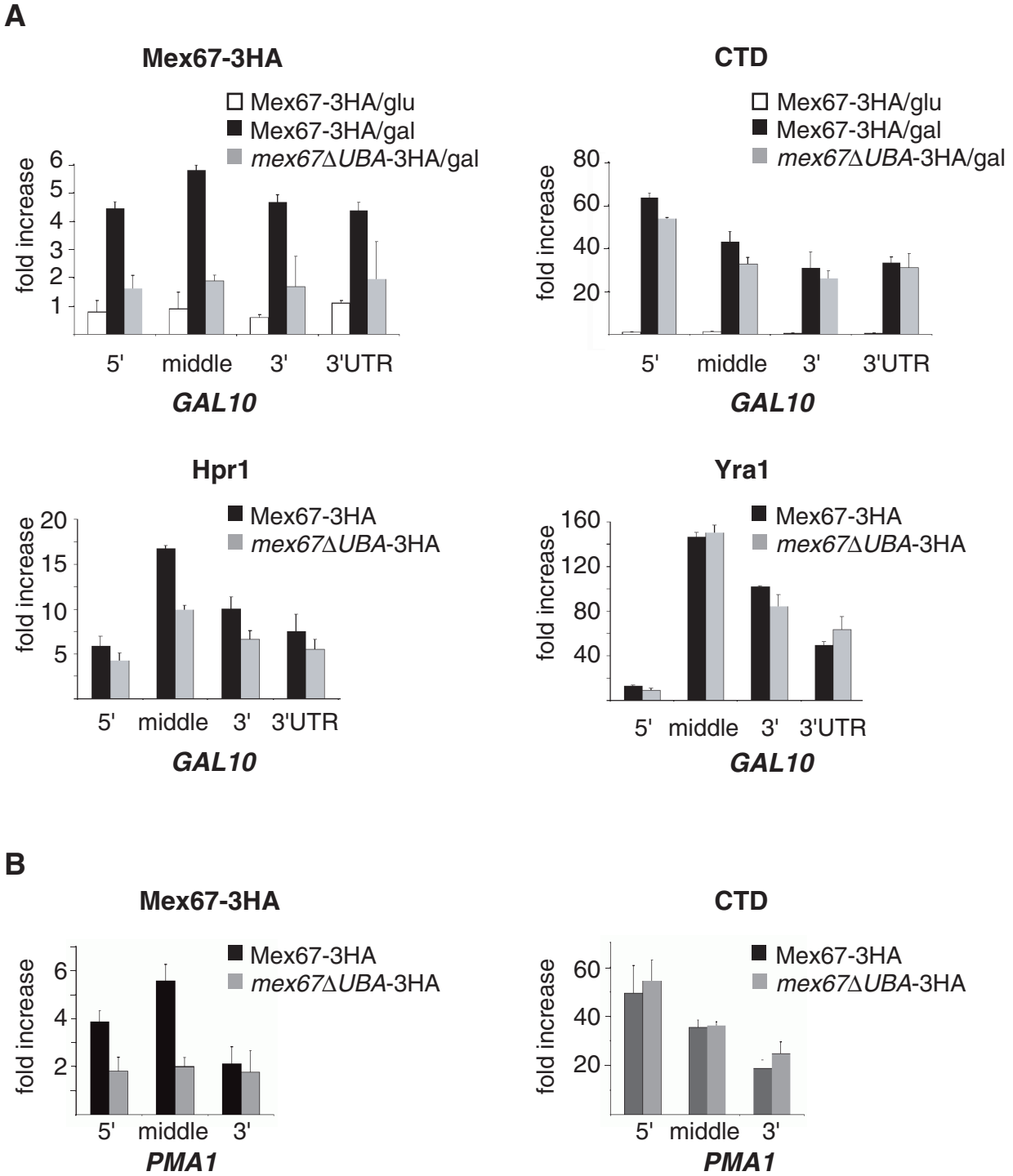
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Mex67-3HA/glu
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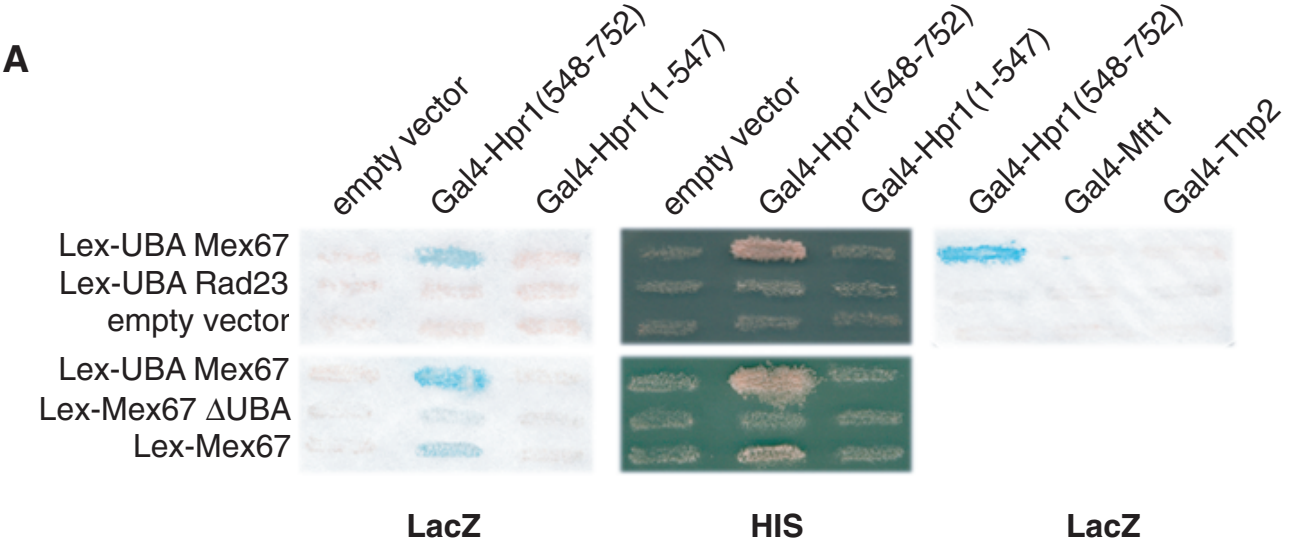
mex67ΔUBA-3HA/gal
fold increase
6
5
4
3
2
1
5'
middle
3'
3'UTR
GAL10
CTD
Mex67-3HA/glu
Mex67-3HA/gal
mex67ΔUBA-3HA/gal
fold increase
80
60
40
20
5'
middle
3'
3'UTR
GAL10
Hpr1
Mex67-3HA
mex67ΔUBA-3HA
fold increase
20
15
10
5
5'
middle
3'
3'UTR
GAL10
Yra1
Mex67-3HA
mex67ΔUBA-3HA
fold increase
160
120
80
40
5'
middle
3'
3'UTR
GAL10
B
Mex67-3HA
Mex67-3HA
mex67ΔUBA-3HA
fold increase
6
4
2
5'
middle
3'
PMA1
CTD
Mex67-3HA
mex67ΔUBA-3HA
fold increase
60
40
20
5'
middle
3'
PMA1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Gal4-Hpr1(1-547)
Gal4-Hpr1(548-752)
Gal4-Mft1
Gal4-Thp2
Lex-UBA Mex67
Lex-UBA Rad23
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Lex-Mex67 ΔUBA
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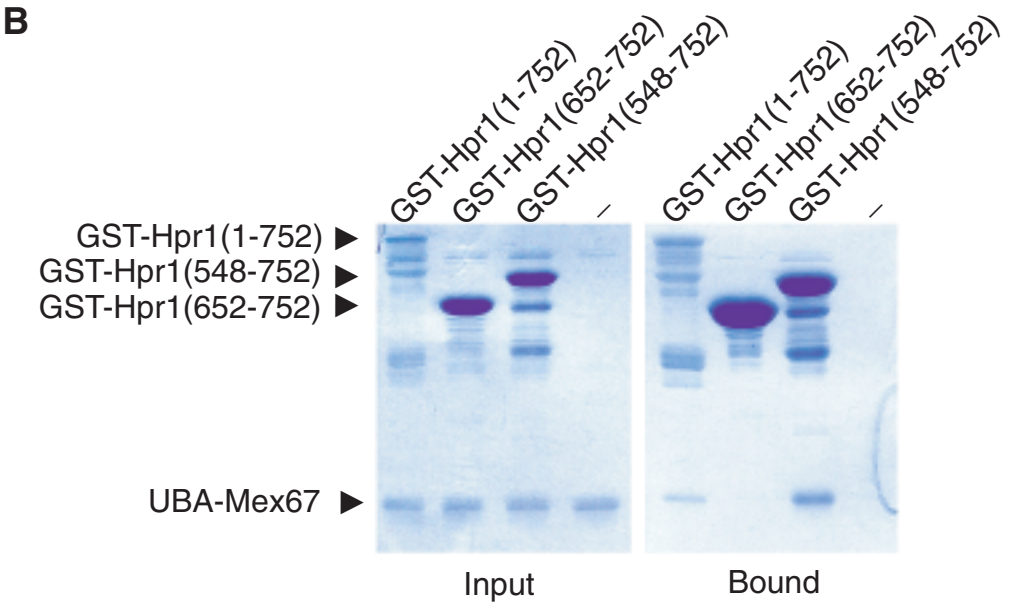
LacZ
HIS
LacZ


B
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GST-Hpr1(652-752)
GST-Hpr1(548-752)
–
GST-Hpr1(1-752)
GST-Hpr1(652-752)
GST-Hpr1(548-752)
–
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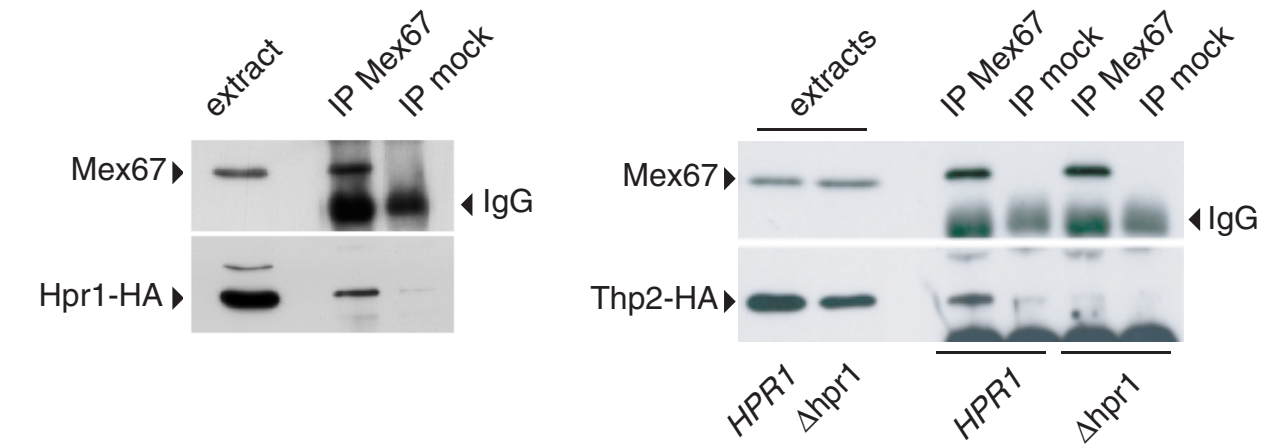
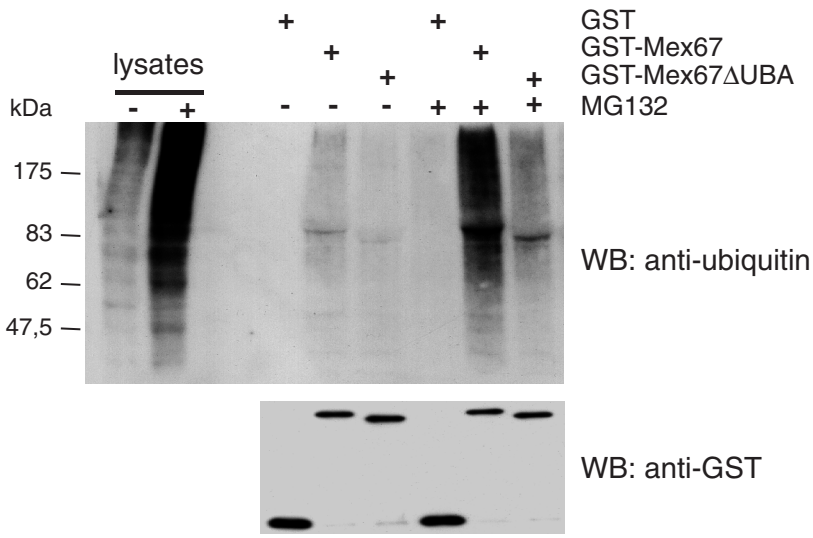
GST-Hpr1(548-752)
GST-Hpr1(652-752)
UBA-Mex67
Input
Bound


C
extract
IP Mex67
IP mock
Mex67
IgG
Hpr1-HA
extracts
IP Mex67
IP mock
IP Mex67
IP mock
Mex67
IgG
Thp2-HA
HPR1
Δhpr1
HPR1
Δhpr1

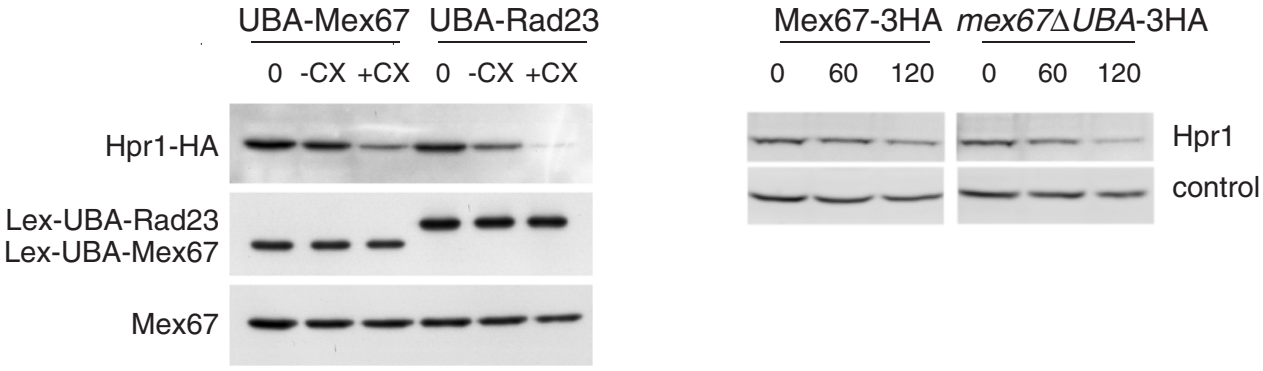
A



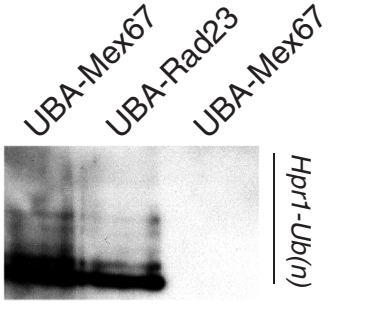
B

Proteins	Kd for mono-ubiquitin (μM)	Kd for tetra-ubiquitin (μM)
Rad23-6His	8.7+/-1.4	1.7+/-0.23
GST-Mex67/Mtr2-6His	22.7+/-4.2	5.9+/-0.7
GST-Mex67ΔUBA/Mtr2-6His	>500	400-500
Mtr2-6His	No binding	No binding

C



D

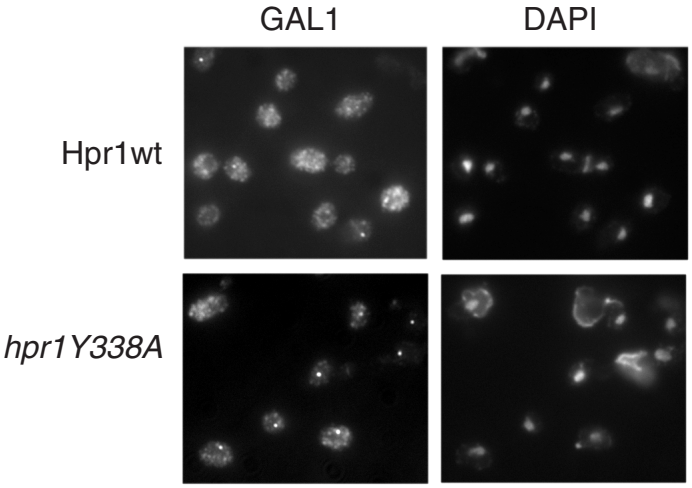


cim3.1/Hpr1-HA (37°C)

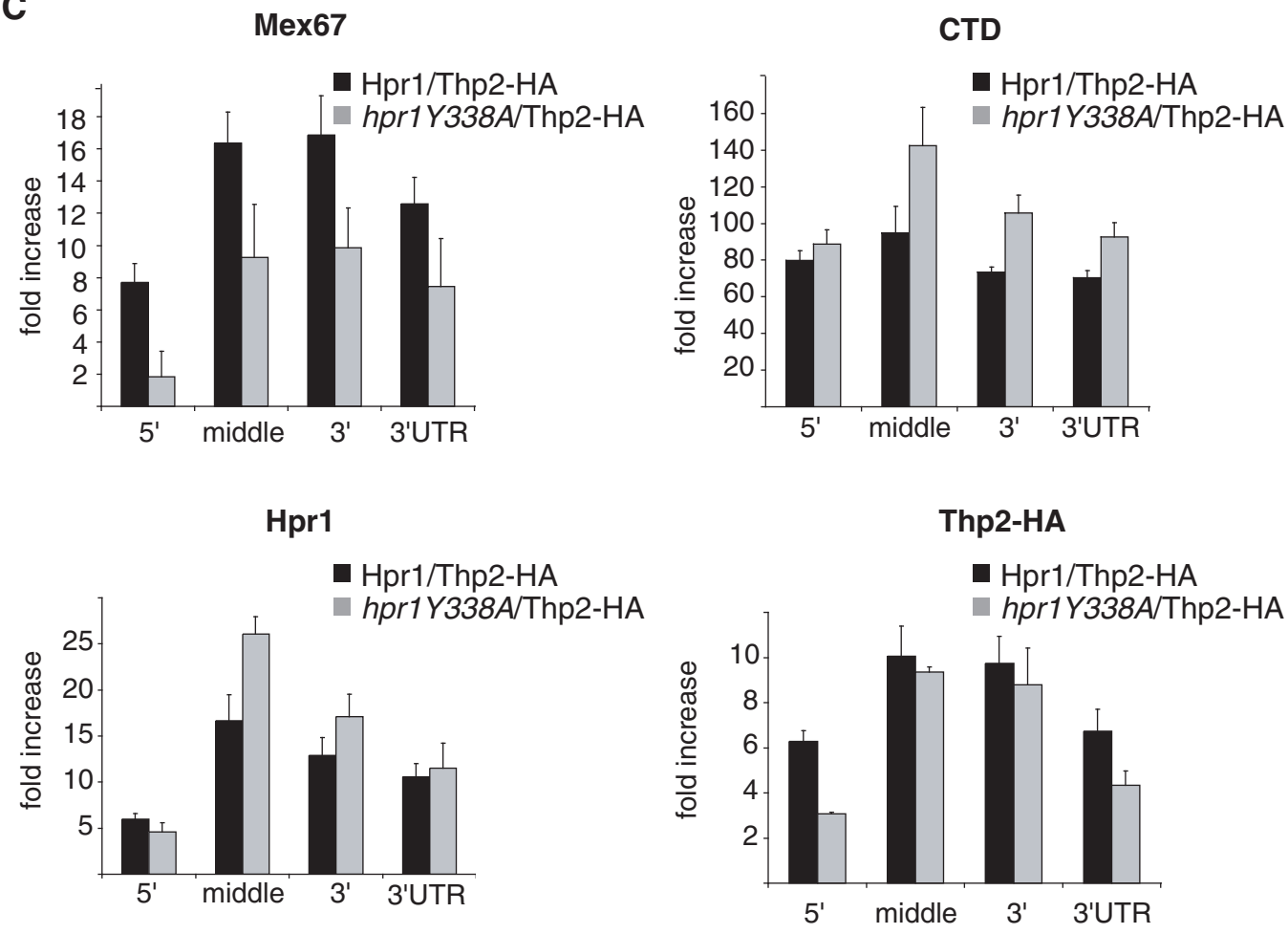
A

Rsp5-binding site	Strain	Half-life of Hpr1
^{335}LPVY^{338}	Hpr1wt	15 min
^{335}LPVA^{338}	*hpr1Y338A*	30 min

B



C



Supplementary informations

Plasmids and Cloning. The DNA fragment encoding the 56 amino acid (543-599) UBA domain of Mex67 and the 56 amino acid (342-398) UBA domain of Rad23 were amplified by PCR using genomic DNA as a template and cloned as BamHI-SalI fragments into pLex10 (Invitrogen), and then subcloned in the BamHI-SalI sites of PET28a (Novagen). DNA encoding Lex-UBA-Mex67 or Lex-UBA-Rad23 fusion proteins expressed under the control of a constitutive or a galactose inducible promoter was obtained by digestion of pLex10-UBA-Mex67 or pLex10-UBA-Rad23 and subcloned into HindIII-SalI sites of p426 ADH (2μm, URA3) or p426 GAL1 respectively (42). Full-length *MEX67* was subcloned as an EcoRI fragment from pGEX4T-1-MEX67 (11) into pLex10 vector, in frame with the DNA binding domain of LexA. A KpnI-BamHI DNA fragment encoding amino acids 513-542 of Mex67 was amplified by PCR and cloned into the same restriction sites of pLex10-MEX67 (1-513) to generate the pLex10-mex67ΔUBA (1-542) construct. DNA encoding GST-Mex67ΔUBA was obtained by digestion of pLex10-mex67ΔUBA and subcloned into EcoRI-SalI sites of pGEX4T-1. For the Hpr1ΔC-ter (1-547) construct, two DNA fragments encoding amino acids 1-373 (BamHI-EcoRV) and 373-547 (EcoRV-SalI) of Hpr1 were obtained by digestion of the pGEX4T-1-Hpr1 vector and by PCR respectively. Both fragments were cloned into BamHI-SalI sites of pADGAL4/2.1 vector (Stratagene), in frame with the GAL4 activator domain. A EcoRI DNA fragment encoding amino acids 548-752 or 652-752 of Hpr1 was amplified by PCR and cloned into the same restriction site of pGEX-4T1. To generate pRS314-MEX67-3-HA and pRS314-mex67-ΔUBA-3-HA, a EcoNI-NheI DNA fragment encoding amino acids 494-599 or 494-542 respectively followed by three HA tags was subcloned into the same restriction sites in the pRS314-mexΔC1 plasmid (7). These plasmids were transformed into the MEX67 shuffle strain (*mex67::*HIS3, pURA3-MEX67, (7)) prior to selection on 5-FOA plates. Expression levels of Mex67-3HA and Mex67-ΔUBA-3HA in the

shuffle strains analyzed by Western blotting with anti-HA antibodies were found similar. Plasmid YEplac181 (2μm, LEU2) containing the coding sequence of Hpr1 +/- 500 bp was previously described (11). Site directed Hpr1 PY domain mutants were constructed by replacing tyrosine 338 by alanine using PCR and confirmed by DNA sequencing. The plasmids HPR1-wt and *hpr1-Y338A* were introduced into the *Δhpr1* strain (11) to generate Hpr1-wt and *hpr1-Y338A* strains. Expression levels of wild type and mutant Hpr1 were analyzed by Western blotting with anti-Hpr1 antibodies and found to be similar.

Chromatin immunoprecipitation (ChIP) analysis. ChIPs were performed as described previously (11, 44) except that cells were cross-linked by incubation with 1.2% formaldehyde for only 10 min. The role of the UBA domain in Mex67 recruitment to *GAL10* was addressed by comparing extracts from the Mex67-3HA and *mex67ΔUBA*-3HA shuffled strains. The cells were grown in YEP 2% raffinose to OD_{600}=1, and induced by the addition of one volume YEP 4% galactose for 2h at 25°C prior to cross-linking. The role of the Hpr1-PY domain in Mex67 recruitment to the *GAL10* gene was analyzed by comparing extracts from Hpr1wt/Thp2-HA and *hpr1Y338A*/Thp2-HA strains pre-grown in Leu- 2% raffinose at 25°C to OD_{600}=1, induced by the addition of one volume Leu- 4% galactose, for 1h at 25°C prior to cross-linking. Sonicated extracts from both experiments were immunoprecipitated with antibodies against HA (16B12 from Covance), RNA polymerase II C-terminal domain (CTD ; 8W16G from Covance), Mex67 and Hpr1 as indicated. Each immunoprecipitation was repeated at least from three different extracts. Immunoprecipitated DNA was analysed by quantitative PCR using the following *GAL10* primer pairs:

GAL10 5' (OFS1061 5'CACTGTGGTAGAGCTAATTGAGAATGG3', OFS1062 5'AAGACCTCTAACCTGGCTACAGAATC3'),

GAL10 middle (OFS1063 5'CCAGTTAAGGGGTGTCGAGGC3', OFS1064 5'AAATTGGCAAACGTGGCTTGAAATC3'),

GAL10 3' (OFS1065 5'CAAGACAAGGTTTTGCAATTGAGCC3', OFS1066 5'CAATCTTGGACCCGTAAGTTTCACC3')

GAL10 3'UTR (OFS1067 5'TGTAGGGACCGAATTGTTTACAAGTTC3', OFS1068 5'GTTGCTACCGTCCATATCTTTCCATAG3').

To calculate the fold increase in signal in the different *GAL10* regions, the absolute values obtained by quantitative PCR were normalized to the values obtained with the non-transcribed intergenic region.

The role of the UBA domain in Mex67 recruitment to *PMA1* was addressed by comparing extracts from the Mex67-3HA and *mex67ΔUBA*-3HA shuffled strains. Cells were grown at 25°C in YEP 2% glucose to OD_{600}=1 prior to cross-linking. Sonicated extracts were immunoprecipitated with antibodies against HA or RNA polII CTD. Each immunoprecipitation was repeated at least from at least three different extracts. Immunoprecipitated DNA was analysed by quantitative PCR as described above using the following *PMA1* primer pairs: PMA1 5' (OFS705 5'TCAGCTCATCAGCCAACTCAAG3', OFS706 5'CGTCGACACCGTGATTAGATTG3'), PMA1 middle (OFS741 5'TTGCCAGCTGTCGTTACCAC3', OFS742 5'TCGACACCAGCCAAGGATTC3'), PMA1 3' (OFS708 5'TACTGTCGTCCGTGTCTGGATCT3', OFS709 5'CCTTCATTGGCTTACCGTTCA3').

Supplemenrary TABLE 1. Yeast strains used in this study.

Name	Background	Genotype	Reference or source
WT	W303	*MATα ade2 his3 leu2 trp1 ura3*	
Hpr1-HA	W303	*MATα ade2 his3 leu2 trp1 ura3*, *HPR1-3HA::TRP1*	(24)
Thp2-HA	W303	*MATα ade2 his3 leu2 trp1 ura3*, *THP2-3HA::TRP1*	This study
Δhpr1-Thp2-HA	W303	*MATa ade2 his3 leu2 trp1 ura3*, *hpr1*::*TRP1*, *THP2-3HA::HIS3*	(11) and this study
Hpr1-HA	DF5	*MATα his3 leu2 lys2 trp1 ura3*, *HPR1-3HA::TRP1*	This study
Mex67-3HA	RS453	*MATa ade2 his3 leu2 trp1 ura3 mex67*::*HIS3* (pRS314-*TRP1-MEX67-3HA*)	(7) and this study
mex67ΔUBA-3HA	RS453	*MATa ade2 his3 leu2 trp1 ura3 mex67*::*HIS3* (pRS314-*TRP1-mex67ΔUBA-3HA*)	(7) and this study
L40		*MATa ade2 his3 leu2 trp1 LYS2::lexA-HIS3* URA3::lexA-lacZ	Gift from J. Camonis
Δerg6 (RH3622)	S288c	*MATa bar1 his4 leu2 ura3 erg6*::*LEU2*	(48)
cim3-1/Hpr1-HA	S288c	*MATα ura3 leu2 his3 cim3-1*, *HPR1-3HA::Kan*^{R}	(49) and this study
Hpr1wt	W303	*MATa ade2 his3 leu2 trp1 ura3*, *hpr1*::*TRP1* (YEplac181-*LEU2-HPR1*)	(11) and this study
hpr1Y338A	W303	*MATa ade2 his3 leu2 trp1 ura3*, *hpr1*::*TRP1* (YEplac181-*LEU2-hpr1-Y338A*)	(11) and this study
Hpr1wt/Thp2-HA	W303	*MATa ade2 his3 leu2 trp1 ura3, hpr1*::*TRP1*, *THP2-3HA::HIS3*, (YEplac181-*LEU2-HPR1*)	(11) and this study
hpr1Y338A/Thp2-HA	W303	*MATa ade2 his3 leu2 trp1 ura3*, *hpr1*::*TRP1*, *THP2-3HA::HIS3*, (YEplac181-*LEU2-hpr1-Y338A*)	(11) and this study
Hpr1-HA-Kan^R (YFS 1748)	W303	*MATa ade2 his3 leu2 trp1 ura3*, *yra1*::*HIS3*, (YCplac33-*URA3-YRA1*), *HPR1-3HA::Kan*^{R}	(11) and this study